PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [4]: A61K 47/00, G01N 33/531	A1	(11) International Publication Number: WO 89/ 06976
		(43) International Publication Date: 10 August 1989 (10.08.89)

(21) International Application Number: PCT/GB89/00093

(22) International Filing Date: 1 February 1989 (01.02.89)

(31) Priority Application Number: 8802174

(32) Priority Date: 1 February 1988 (01.02.88)

(33) Priority Country: GB

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(81) Designated States: AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent), FR (European patent), GB, GB (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), SU, US.

Published
With international search report.
With amended claims.

(54) Title: METHOD OF DRYING MACROMOLECULES

(57) Abstract

A method of drying a biological macromolecular substance by drying an aqueous solution or suspension thereof, optionally in the presence of a stabilising material, is characterised in that the aqueous solution or suspension is formulated in the presence of one or more efflorescent alkali metal, ammonium or alkaline earth metal salts and then dried.

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Method of drying macromolecules

This invention relates to an improved process for drying biological materials, e.g. proteins and other macromolecules, viruses etc. In particular the invention concerns a method of obtaining a dried product which will remain dry without being hermetically sealed or otherwise carefully stored. The invention also relates to dried products produced by such a method.

Various methods for drying biological materials have been published. Lyophilisation of some biological materials from solutions containing various sugars has been described in GB 2009198A, GB 2126588A and JP 58074696A. The sugars include trehalose, glucose, galactose. Our earlier PCT/GB86/00396 describes the use of trehalose to stabilise such materials against drying at ambient and elevated temperatures. In addition, our UK Patent Application 88 01338 discloses the use of trehalose in the stabilisation of live viruses against freezing and drying, either frozen or at ambient temperatures, and our UK Patent Application 8715238 discloses the inclusion of trehalose in food preparations. It is thus now possible to dry safely a whole range of biological materials including

immunological components, viruses, enzymes and fluorescent agents and proteinaceous or other food stuffs.

A frequent, almost inevitable, problem with this technique is that such preparations require storage under anhydrous conditions in impermeable containers with a desiccant. This is necessary because both the macromolecules e.g. antibodies, enzymes and the like and also the carbohydrate molecules e.g. trehalose, sucrose etc. are hygroscopic when dried. In addition, the ionic buffers present usually contain deliquescent salts. All these factors combine to enhance the imbibition of water from a humid environment. This can result in the desiccated macromolecules being exposed to extremely hypertonic aqueous conditions whenever packs of dried material are opened in a humid atmosphere.

This means that such preparations are vulnerable to imperfect storage conditions and their stability can be compromised by sub-optimal handling. This occurs frequently in practice because of the tradition of keeping biologically active molecules under refrigeration. This causes condensation of atmospheric water on the cold preparation whenever the refrigerator door is opened. The preparations are also very unstable in humid or tropical climates.

This invention provides a simple method which relies on molecular mechanisms to remove imbibed water from dried macromolecular preparations, especially those protected by a sugar from desiccation damage.

According to this invention there is provided a method of drying a biological macromolecular substance by drying an aqueous solution or suspension thereof, optionally in the presence of stabilising material, characterised in that the aqueous solution or suspension is formulated in the presence of one or more efflorescent alkali metal, ammonium or alkaline earth metal salts and then dried.

The method depends on replacement of the deliquescent salts, such as sodium chloride, in conventional buffers/osmotic pressure regulators with efflorescent salts such as sodium sulphate The term "efflorescent" is used herein to refer to salts which lose water under ambient humidity, e.g. those salts having a vapour pressure of water of crystallization of at least 15mm Hg (2000 Pa). Various salts can be used depending on the nature of the material to be dried. Naturally, the salt must be compatible with the material to be dried. For food applications the approved additive calcium lactate is efflorescent and suitable. Other efflorescent salts which might find application in certain formulations include:

1. Sodium salts: acetate trihydrate, tetraborate decahydrate (borax), bromoiridite dodecahydrate, carbonate heptahydrate, metaperiodate trihydrate, metaphosphate(tri)hexahydrate, hydrogen orthophosphate dodecahydrate, sulphite heptahydrate, thiosulphate pentahydrate (hypo).

2. Non-sodium salts (where applicable): calcium lactate hydrate, magnesium salicylate tetrahydrate, magnesium sulphate heptahydrate (Epsom salts) and ammonium sulphate.

In preparations which do not contain an added buffer, e.g. foodstuffs such as egg and milk, the efflorescent salt can be added as such, without the need to replace the buffer salt, provided that care is taken to maintain appropriate molarities.

While we do not intend to be bound by theoretical considerations, it is believed that the action of the efflorescent salt is explained as follows:

Sodium sulphate crystallises from aqueous solutions as the decahydrate $Na_2SO_4.10H_2O$ (Glauber's salt). Because the vapour pressure of the water of crystallisation is higher than the vapour pressure of atmospheric water even in very humid environments, this

salt actively "pumps" water from the crystals into the atmosphere. Any water imbibed by the hygroscopic desiccated preparation containing sodium sulphate crystals, is taken up by this soluble salt and in turn is expelled by the efflorescence phenomenon into the atmosphere. Thus the efflorescent salt which exists in dried preparations in a microcrystalline form, homogeneously distributed throughout the dry preparation, acts as a "molecular water pump". This maintains dried preparations in a dry state under conditions of frequent exposure to humid conditions.

When dissolved in an excess of water, sodium sulphate gives a solution of neutral pH and is non reactive in solution and non toxic to cells or tissues. A 0.12M solution is roughly isotonic with mammalian serum. It substitutes in all significant respects for the physiological salt sodium chloride.

In cases where the added salt is highly efflorescent, it is possible that the molecular water pump action will actually decrease the residual water content of the "dried" preparation. For example agarose gels containing sodium sulphate instead of sodium chloride can be dried to a clear tough film which, on storage under moderately humid atmospheric conditions, actually continue to lose water, causing blooming of the

surface as minute crystals of anhydrous sodium sulphate form. This process is entirely harmless, and the gel can be completely rehydrated.

To illustrate in practice the operation of the drying mechanism we measured actual water adsorption and desorption in a model system.

Example 1. Quantitation of water adsorption by dried protein films under controlled humidity in the presence of hygroscopic and efflorescent buffers.

Method

A 10% w/v solution of salt-free bovine plasma albumin (BSA) was made up in either physiological Dulbecco's phosphate-buffered saline (PBS) or in 0.12M sodium sulphate solution. Samples of about 1ml of solution were spread in pre-weighed sterile polystyrene tissue culture grade Petri dishes (Nunclon 60 x 15mm Cat No.1-50288) and dried by being left open in a warm room (37°C) for 48hr. They were then weighed on a 5 decimal place Mettler HK60 electronic microbalance to obtain the dry weight of protein + buffer.

They were then sealed into airtight, glass humidity chambers where they were exposed for 60hr to air in which the relative humidity was controlled at 90% with a

standard sulphuric acid solution as detailed in "The Handbook of Chemistry and Physics", 67th Edition, CRC press. They were then removed and immediately reweighed to 5 decimal places to measure the water absorbed.

All samples were then stored in an open container at room temperature and ambient relative humidity for 8 days and weighed again to measure the efficiency with which the absorbed water is desorbed.

Results. (Table I)

These show that at 90% RH much is absorbed by the dried protein. Much more is absorbed in PBS buffer (30.3%) than in sodium sulphate buffer (13.4%).

Note that the ratio of BSA to buffer in this experiment (about 1:1.7 w/w) was much higher then usually present in reagent formulations (about 1:17 w/w) so that the efficiency of the water removed by the efflorescent buffer may have been sub-optimal. In addition no trehalose or other humectant was present.

TABLE 1

Buffer	Total dry weight (mg)	Total humid weight (mg)	Water gain (%)
P.B.S	222.0	289.2	30.3
Na_2SO_4	314.7	356.9	13.4

Example 2

A second type of quantitative experiment was set up with the fluorescent reagent R-Phycoerythrin (RPE) at the clinically relevant concentration of 1mg/ml in 10% w/v trehalose in either PBS or 0.12M sodium sulphate solution. The experiments were done as in the previous example.

The results (table II) show that at 90% relative humidity a large amount of water was imbibed by the dried RPE in PBS buffer where more than 20% of the dry weight of RPE was absorbed. This was not the case in sulphate buffer where only 2.4% of water was imbibed. There was a dramatic difference in the appearance of the two preparations. In the PBS buffer the RPE became liquefied and syrupy in consistency while in the sulphate buffer the dried RPE remained dry and

non-tacky. Within 4 hr of removal from the humidity chamber and storage at ambient humidity in room air, the imbibed water was desorbed from the PBS preparation to a level of 3.5-3.9%. During this process a marked phase separation occurred with large crystals of buffer separated out leaving highly concentrated syrupy protein collections in the petri dish. It is this repeated liquefaction and drying with phase separation which causes damage to the desiccated protein.

TABLE 2

Buffer	Total dry weight (mg)	Total humid weight (mg)	Water gain (%)
P.B.S	211.8	257.9	21.8
Na_2SO_4	245.9	251.8	2.4

Example 3

As an example of the efficiency of this buffer system in assay kits we chose a dried preparation of a readily quantitated immunoassay system with which it was possible to measure precisely any loss of activity under sub-optimal storage conditions. The reagents consisted of a conjugate of the biotin-binding protein

Streptavidin, (purified from Streptomyces avidinii), with the fluorescent phycobiliprotein R-Phycoerythrin. This conjugate was dried in flat bottomed wells of a 96-well microtitre plate in buffers of various formulations containing the disaccharide trehalose as a means of completely preventing the loss of conjugate activity on drying.

The buffer formulations used were various ratios of standard Phosphate-buffered saline and 0.1M sodium sulphate ranging from 90% of the former plus 10% of the latter to 90% sodium sulphate buffer and 10% PBS.

Plates containing these preparations were dried in a warm room at 37°C for 24 hr. Samples of these plates were then stored at 37°C or at room temperature or at 4-10°C in a standard refrigerator for 2 months.

The dried preparations were tested for their ability to stain rat lymphocytes labelled with saturating amounts of a biotin-labelled monoclonal mouse anti-rat CD4 antibody W3/25, (D.W.Mason, R.P.Arthur, M.J.Dallman, J.R.Green, G.P.Spickett & M.L.Thomas. 1983, Immunological Reviews, 74 pp.57-82). The stained preparations were produced by rehydrating the wells of the microtitre plates with 50µl distilled water and then adding a suspension of 10^6 rat lymph-node

lymphocytes to each well. The lymphocytes were suspended in complete RPMI 1640 tissue culture medium containing 10% Haemaccel as a protein supplement (TCM). Staining for 30 min at 4°C was followed by two washes in TCM before analysis in a FACS 420 Fluorescence activated cell sorter (Becton Dickinson, Sunnyvale, Calif. USA) operating an argon laser at the 488 nm line at a power of 300 mW.

The intensity of staining of these lymphocytes with the R-Phycoerythrin/Streptavidin conjugate is a direct measure of the activity of the conjugate. The fluorescence intensity is recorded as the logarithm of the brightness on the X axis against the number of cells on the Y axis. Thus any decrease in activity is revealed as a shift to the left of the modal value (peak) of the brightly fluorescent cells.

Results:-

When stored for 2 months either at room temperature or at 4-10°C in a refrigerator (Fig 1a, b) there was considerable loss of activity in buffers containing smaller amounts of 0.1M sodium sulphate buffer. This loss of activity was inversely proportional to the amount of sulphate buffer present. In 90% sulphate buffer there was no loss at all. In 50% sulphate buffer the conjugate became almost completely inactive (figs 1 and 2).

In contrast, where the conjugates were stored at 37°C in a dry warm room (Fig. 1c) the buffer formulation had little effect. There was minimal loss of activity in any of the buffer formulations.

Thus the preservation of activity in the conjugates was dependent on preservation of the dry state. This could be achieved by maintaining the conjugates in a dry environment such as the warm room or by using a high concentration of sodium sulphate buffer which maintained the dried conjugate in the dry state by efflorescence even in humid conditions.

Example 4

To show the application of this technique to a commercial product, an instant two colour immunofluorescence assay was formulated in the dry state by mixing 25µl of a solution of R-Phycoerythrin conjugated to the mouse IgGi monoclonal anti rat CD4 antibody W3/25 and 25µl of a solution of fluorescein conjugated to the mouse IgGi anti rat CD8 antibody MRC OX-8 (both dissolved in 0.1M sodium sulphate solution) was 50µl of 10% trehalose in distilled water in the wells of a microtitre plate and drying at 37°C. This plate was kept at room temperature for 2 months and the ability of the dry conjugates to give bright 2 colour

immunofluorescence staining with complete, specific staining of the relevant cell populations was recorded. In comparison with antibodies dried without trehalose which were almost inactive, the antibodies dried with trehalose were as good in all respects, including potency as fresh non-dried antibodies (fig. 2a, b, c).

LEGENDS

Figure 1

FACS 420 histograms of red fluorescence of Streptavidin/ R-Phycoerythrin (SA/RPE) conjugate staining of W3/25 labelled rat lymphocytes. The SA/RPE conjugates were dried at 37°C in the wells of flat-bottomed microtitre plates in buffers which consisted of various mixtures of 0.1M sodium sulphate and PBS. Thereafter they were stored either at room temperature, 4-10°C in a refrigerator or at 37°C in a warm room for 2 months before use for staining.

1a. Storage at room temperature: There is progressive loss of staining potency as the proportion sulphate buffer is reduced. Curves for 90%, 70% and 50% buffers are shown. With lower proportions of sulphate buffer the conjugates were completely inactive. Storage in 90% buffer retains staining potency equal to that of fresh SA/RPE conjugate.

1b Storage at 4-10°C: Again the potency of the conjugates is greatly reduced at 50% and 70% sulphate concentration while 90% sulphate buffer retains full potency.

1c Storage at 37°C: When stored in a dry atmosphere, high proportions of sulphate buffer are not necessary to preserve staining potency.

Figure 2 Simultaneous 2-colour immunofluorescence using dried conjugates

Photographs of VDU screens of FACS 420 analysis of mixed W3/25 αCD4 antibody coupled to RPE and MRC OXB αCD8 antibody coupled to FITC on rat lymphocytes. Photographs show fluorescence on X-axis against cell number on Y-axis

Fl 2 = red fluorescence on RPE

Fl 1 = green fluorescence of FITC

Top: fresh conjugate

Middle: conjugate dried in 0.1M sodium sulphate containing 10% trehalose and stored for 1 month at RT° before staining

Bottom: conjugate dried in 0.1M sodium sulphate buffer without trehalose showing loss of staining potency. Sodium sulphate buffer maintains trehalose-dried conjugate with full potency when stored at room temperature but does not substitute for trehalose as a preservative of conjugate function.

Example 5

Gravimetric Tests

Method:

The model system consisted of 1ml 1% bovine serum albumin plus 10% trehalose in 10mM Trishydroxymethyl-aminomethane (Tris) chloride buffer pH 7.4 to which was added the relevant concentration of the test salts. The mixture was dried at 3° for 24hr in hydrophobic plastic dishes preweighed on a top-pan balance to an accuracy of 1 milligram, then reweighed and transferred to sealed chambers in which the relative humidity was controlled at 20-22°C by saturated solutions of various salts as described in "Handbook of chemistry and physics" 67th edition p E-42 R.C. Weast editor (1986) CRC press Paton Fla. USA. At various times the plastic dishes were removed and immediately reweighed. After about 120 hr

they were returned to ambient temperature and relative humidity and reweighed at intervals thereafter until their weight was stable.

The results are plotted as % of original weight at the various weighing times.

Other efflorescent salts tested

1. Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)

Results: Caused protein preciptiation and therefore could not be used in this application.

2. Copper sulphate pentahydrate ($CuSO_4.5H_2O$)

Results: Caused protein precipitation and therefore could not be used in this applicaton.

3. Disodium hydrogen orthophosphate dodecahydrate $Na_2PO_4.12H_2O$.

BSA readily dissolved in 200mM and 300mM solution. Solutions were dried at about 47% RH in an incubator room at 37° and then transferred at 24hr from the 37° room to humidity chambers at 51% RH, 80% RH or 90% RH. There was uptake of water at all 3 RH. This is probably

due to the fact that the dodecahydrate crystals lose water at 37° to the dihydrate which then absorbs water to the heptahydrate form in more humid environments. This is the form that is stable at ambient temperature and humidity. This is supported by the fact that 1ml 300mMolar disodium hydrogen orthophosphate dihydrate theoretically absorbs $1.5x10^{-3}$gm moles of water to form the heptahydrate. This amounts to 27mg which is precisely the increase in weight of the phosphate-dried mixture on equilibration with ambient humidity.

The mixture increased in weight by 50% at 90% RH but only by 20% at 80% RH and about 15% at 51% RH. when transferred back to room air conditions at 144hr the adsorbed water was lost to give a stable weight at about 115-120% of initial 37° weight. Fig. 3.

4. Sodium sulphate decahydrate ($Na_2SO_4.10H_2O$).

BSA readily dissolves in 200mM or 300mM sodium sulphate. When transferred from the 37° room RH to humidity chambers, there was no water uptake at 51% or at 80% RH. There was uptake of 50% of the initial weight only at 90% RH. This water was promptly lost on return to roon air. Fig. 4.

5. Sodium chloride (NaCl). [control, non efflorescent]

Mixture absorbs no water on transfer from 37° to 51% RH. Absorbs 50% of initial weight at 80% RH and 100% original weight at 90% RH. Readily loses water again when returned to room air. Fig. 5.

Summary:

Data is presented as a comparison of chloride, sulphate and phosphate on ach graph at various RH in Figs. 6, 7, 8.

Both disodium hydrogen orthophosphate and sodium sulphate prevent uptake of water by the hygroscopic mixture of BSA and trehalose at 80% RH and reduce the water uptake by 50% at RH of 90% when compared with the neutral (non hygroscopic, non efflorescent) salt sodium chloride. Thus the phosphate buffer is as effective limiting the uptake of water at 90% RH when the water of hydration of the heptahydrate (stable) form of the salt is taken into account.

At 20°C these salts exert an aqueous tension equivalent to a relative humidity of:-

	Aqu tension (MmHg)	RH
NaCl	4.5	35%
$Na_2HPO_4.12H_2O$	16.5	95%
$Na_2SO_4.10H_2O$	16.1	93%

Thus they permit the imbibition of water at relative humidities at or above the vapour pressures of their own water of crystallisation but not below it. for this reason Na_2SO_4 is the ideal salt as it is neutral in solution, non toxic, nonreactive with biological molecules, has a normal salty taste and will protect trehalose-dried biological molecules from moisture damage at relative humidities up to between 80 and 90%.

Disodium hydrogen orthophosphate is another example of an effective salt with a potentially slightly higher protective effect. It suffers from the disadvantage that reducing the pH will lead to the formation of sodium dihydrogen orthophosphate which is slightly deliquescent. If the pH is not adjusted a solution of disodium hydrogen phosphate in water is alkaline with a pH of about 9.5. This is thus not a suitable salt for biological molecules which require a physiological pH of 7.4.

CLAIMS:

1. A method of drying a biological macromolecular substance by drying an aqueous solution or suspension thereof, optionally in the presence of a stabilising material, characterised in that the aqueous solution or suspension is formulated in the presence of one or more efflorescent alkali metal, ammonium or alkaline earth metal salts and then dried.

2. A method according to claim 1 in which the efflorescent salt replaces sodium chloride or another hygroscopic salt as an osmotic pressure regulator and/or buffer in the solution or suspension.

3. A method according to claim 1, in which the efforescent salt is selected from the following sodium salts:- sulphate, acetate trihydrate, tetraborate decahydrate (borax), bromoiridite dodecahydrate, carbonate heptahydrate, metaperiodate trihydrate, metaphosphate(tri)hexahydrate, hydrogen orthophosphate dodecahydrate, sulphite heptahydrate, thiosulphate pentahydrate (hypo), and the following non-sodium salts:- calcium lactate hydrate, magnesium salicylate tetrahydrate, magnesium sulphate heptahydrate (Epsom salts) and ammonium sulphate.

4. A method according to claim 1, in which the efflorescent salt is sodium sulphate or disodium hydrogen phosphate.

5. A method according to claim 1, in which the solution or suspension to be dried contains a sugar as stabilising material.

6. A method according to claim 5, in which the sugar is trehalose.

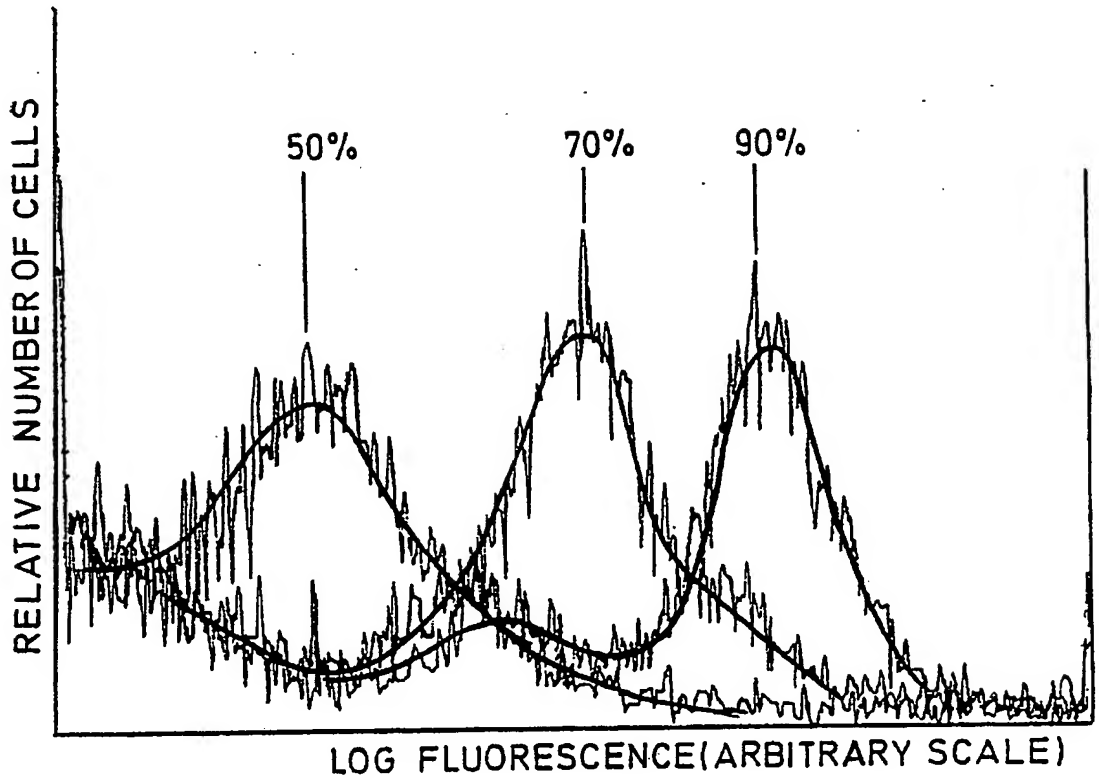
AMENDED CLAIMS

[received by the International Bureau on 21 June 1989 (21.06.89)
claims 1-2 replaced by new claim 1 , claim 3 renumbered claim 2 , claim 3 added (1 page)]

1. A method of drying a biological macromolecular substance by drying an aqueous solution or suspension thereof, containing one or more alkali metal, ammonium or alkaline earth metal salts as buffer or osmotic pressure regulator or the like, optionally in the presence of a stabilising material, characterised in that substantially all the said salts are selected from efflorescent salts.

2. A method according to claim 1, in which the salt is selected from the following sodium salts:- sulphate, acetate trihydrate, tetraborate decahydrate (borax), bromoiridite dodecahydrate, carbonate heptahydrate, metaperiodate trihydrate, metaphosphate(tri)hexahydrate, hydrogen orthophosphate dodecahydrate, sulphite heptahydrate, thiosulphate pentahydrate (hypo), and the following non-sodium salts:- calcium lactate hydrate, magnesium salicylate tetrahydrate, magnesium sulphate heptahydrate (Epsom salts) and ammonium sulphate.

3. A method according to claim 1, in which the salt has a vapour pressure of cystallisation of at least 15mm Hg (2000 Pa).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90%
RELATIVE NUMBER OF CELLS
LOG FLUORESCENCE(ARBITRARY SCALE)

FIG.1a.

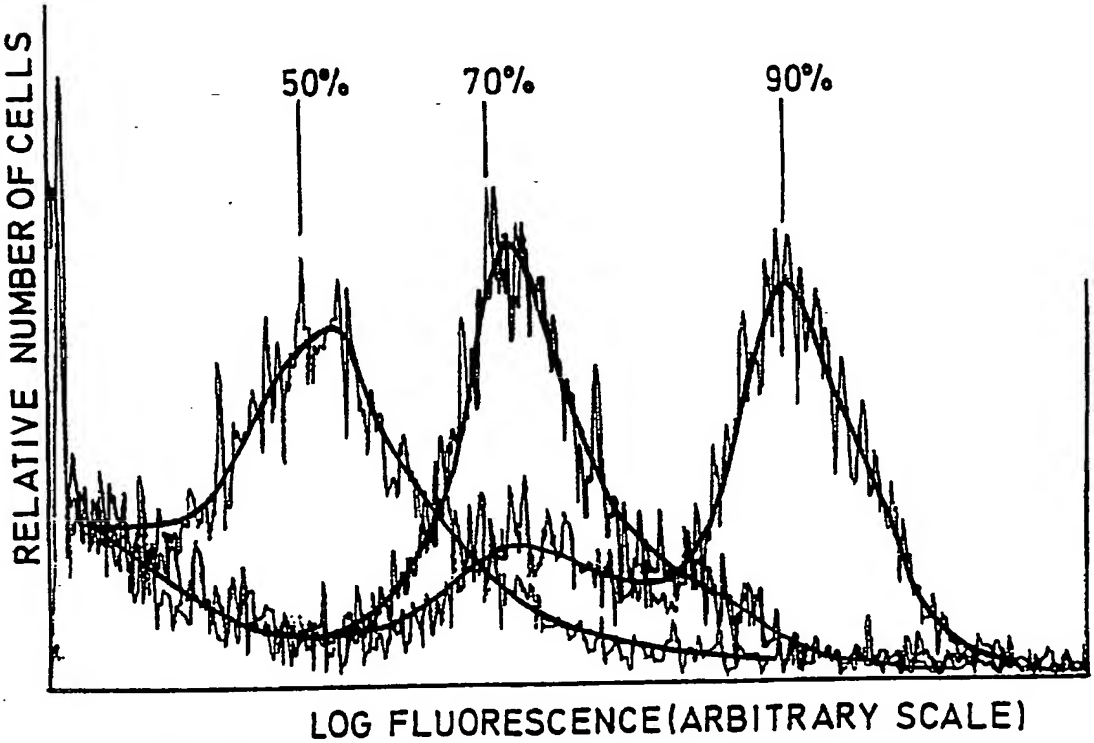
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FIG.1b.

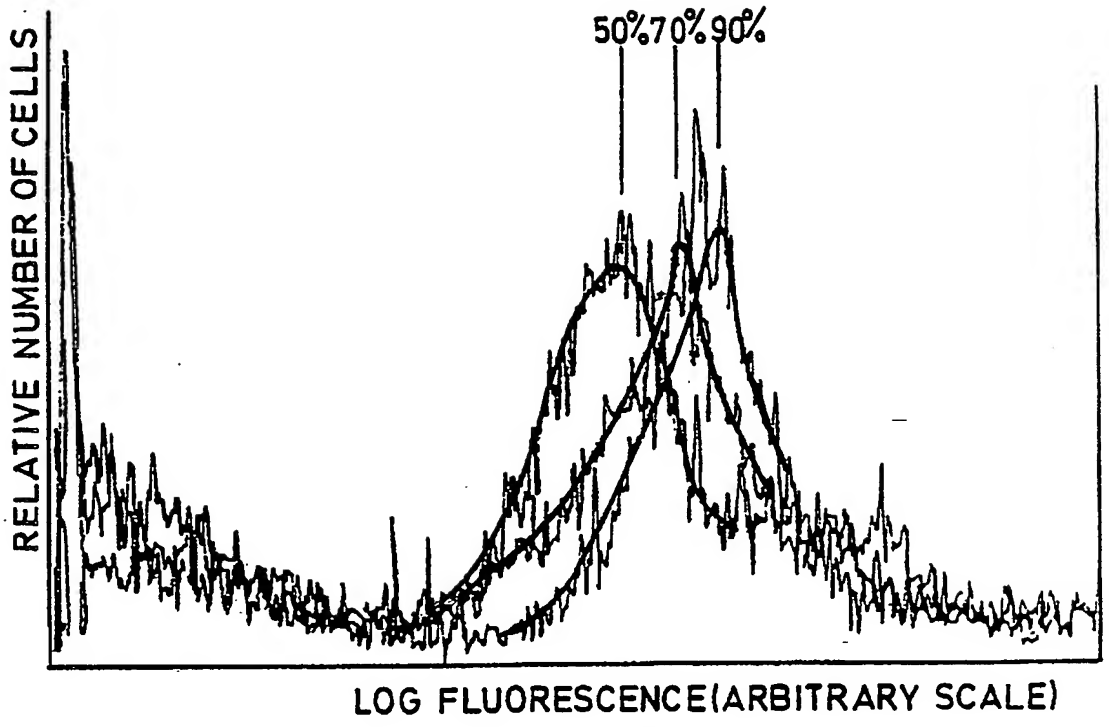
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FIG.1c.

FIG.2a.

FRESH ANTI-CD4 AND ANTI-CD8

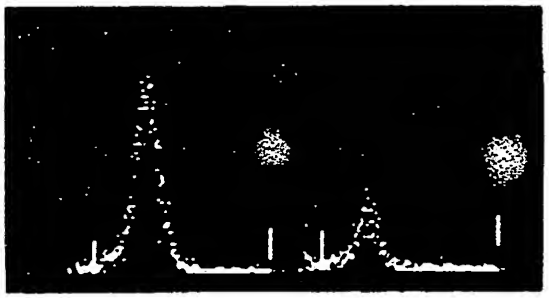

FIG.2b.

DRIED WITH PRESERVATIVE

FIG.2c.

DRIED WITHOUT PRESERVATIVE

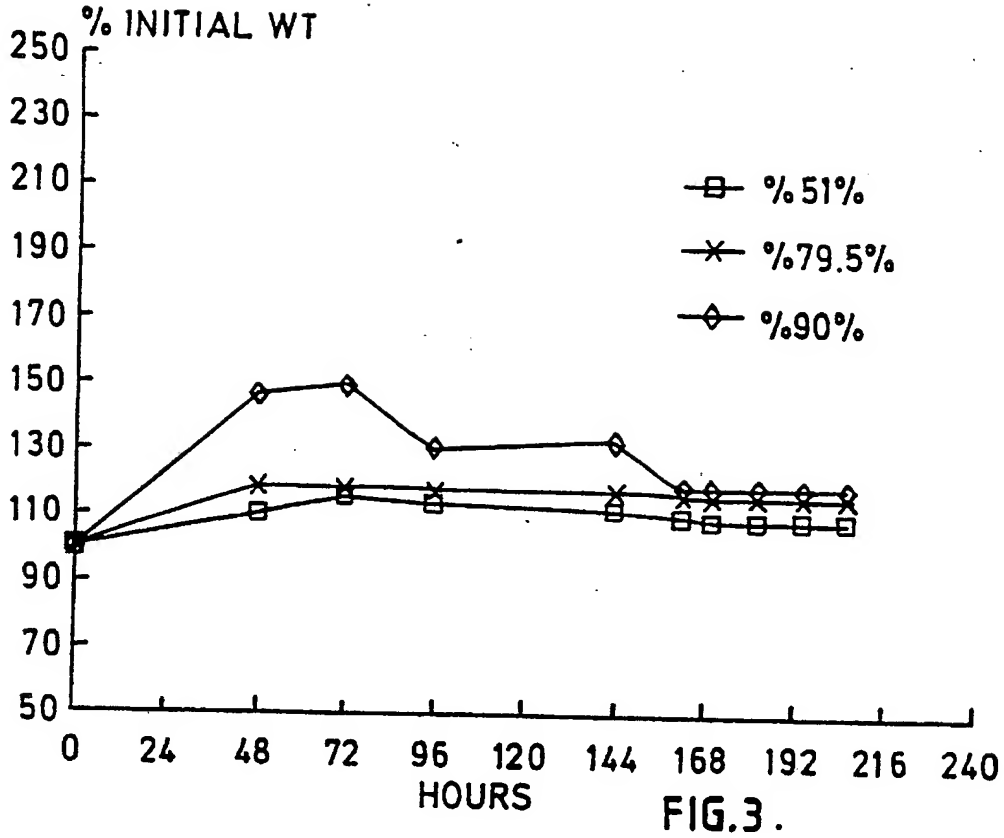
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FIG.3.

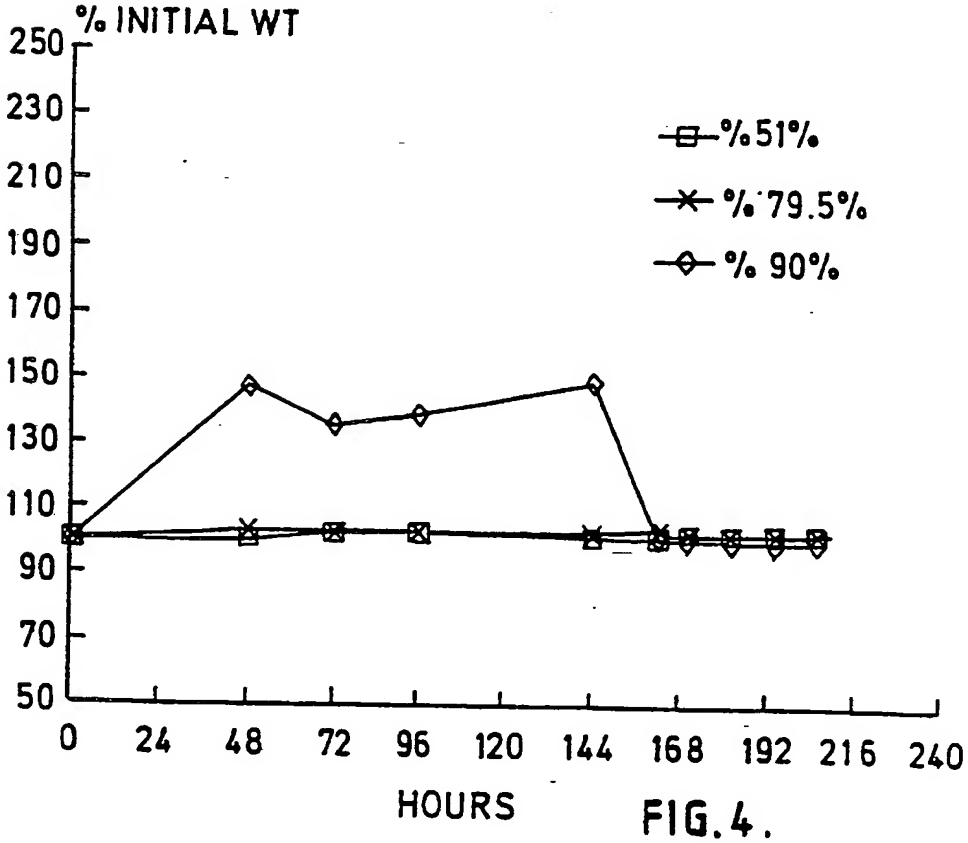
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FIG.4.

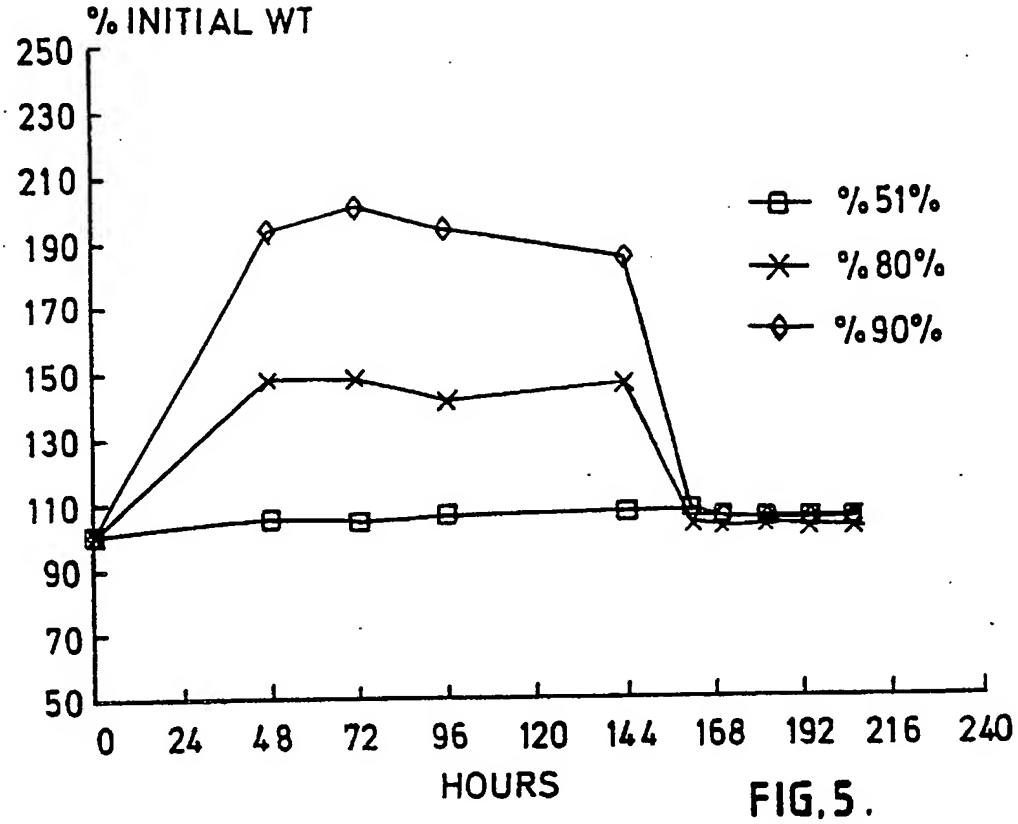
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FIG. 5.

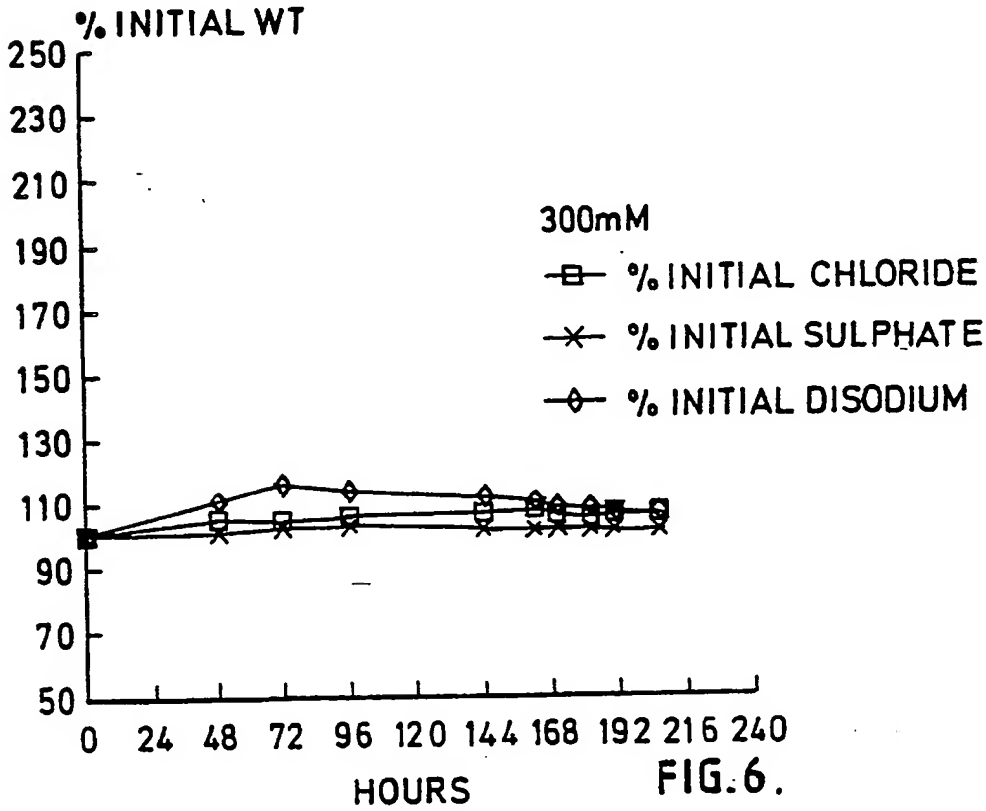
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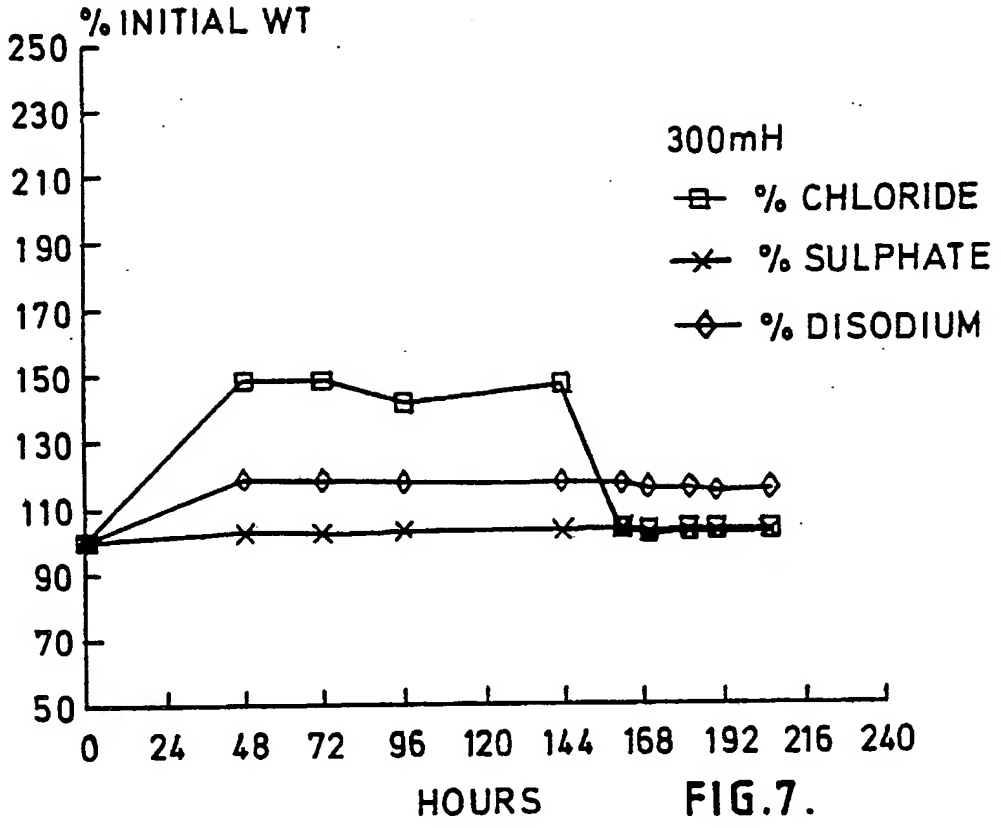
FIG. 6.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HOURS
300mH
% CHLORIDE
% SULPHATE
% DISODIUM

FIG.7.

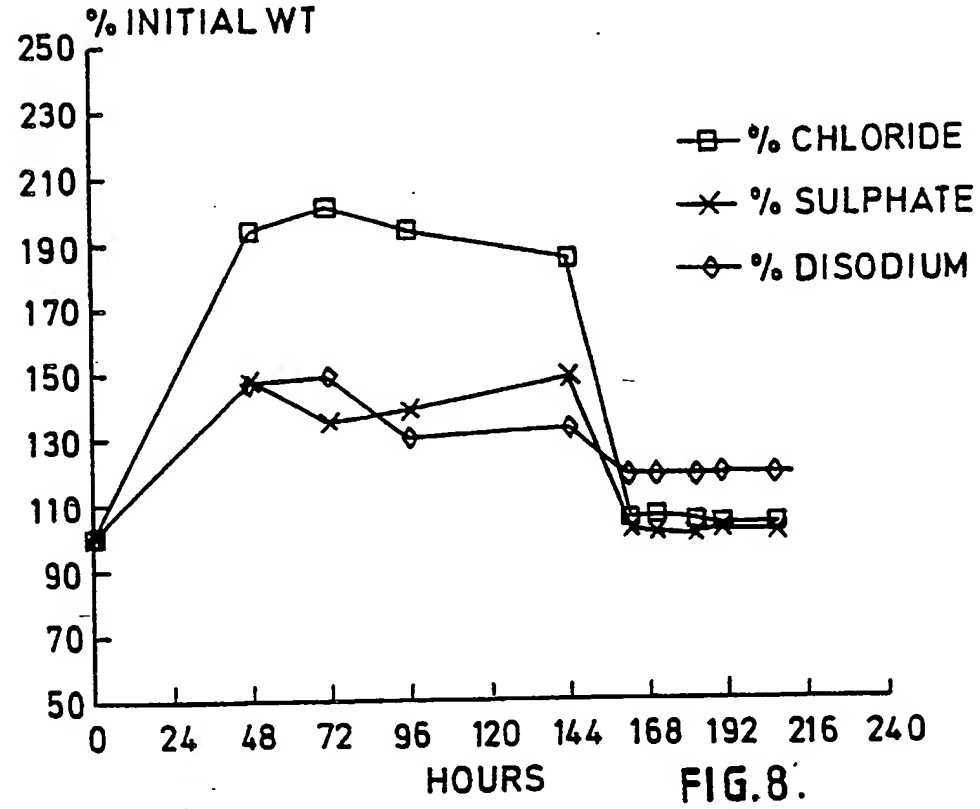
% INITIAL WT
250
230
210
190
170
150
130
110
90
70
50
0
24
48
72
96
120
144
168
192
216
240
HOURS
% CHLORIDE
% SULPHATE
% DISODIUM

FIG.8.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00093

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: A 61 K 47/00, G 01 N 33/531

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC4	A 01 N; A 61 K; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	CH, A5, 622672 (BERINGWERKE AKTIENGESELLSCHAFT) 30 April 1981, see page 3, column 2, line 15 - line 56 --	1-4
X	US, A, 3539450 (A. DEUTSCH) 10 November 1970, see the whole document --	1-5
X	EP, A1, 0192320 (UNILEVER PLC ET AL.) 27 August 1986, see page 4, line 32; claims 1,7 --	1,5,6
X	US, A, 3862302 (R. T. PRICE ET AL.) 21 January 1975, see column 5, line 45 - line 53; abstract --	1,2,5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th April 1989	27 APR 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	CA, A, 1153314 (AMERICAN HOME PRODUCTS CORPORATION) 6 September 1983, see claims 1,4 --	1,2
X	Patent Abstracts of Japan, Vol 11, No 15, P536, abstract of JP 61-189454, publ 1986-08-23 (FUJISAWA PHARMACEUT CO LTD) --	1,2,5
X	EP, A2, 0030199 (MERCK & CO. INC.) 10 June 1981, see page 2, line 27; claim 1 --	1,2,5
X	GB, A, 1575155 (MERCK & CO. INC.) 17 September 1980, see page 2, line 4 - line 5; claim 1 --	1,2

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 89/00093

SA 26727

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 03/03/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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		DE-A-	2546166	28/04/77
		BE-A-	847354	15/04/77
		LU-A-	75987	25/05/77
		AU-D-	18694/76	20/04/78
		US-A-	4157383	05/06/79
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